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Research Paper

BDNF is required for taste axon regeneration following unilateral chorda tympani nerve section

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ABSTRACT

Taste nerves readily regenerate to reinnervate denervated taste buds; however, factors required for regeneration have not yet been identified. When the chorda tympani nerve is sectioned, expression of brain-derived neurotrophic factor (BDNF) remains high in the geniculate ganglion and lingual epithelium, despite the loss of taste buds. These observations suggest that BDNF is present in the taste system after nerve section and may support taste nerve regeneration. To test this hypothesis, we inducibly deleted *Bdnf* during adulthood in mice. Shortly after *Bdnf* gene recombination, the chorda tympani nerve was unilaterally sectioned causing a loss of both taste buds and neurons, irrespective of BDNF levels. Eight weeks after nerve section, however, regeneration was differentially affected by *Bdnf* deletion. In control mice, there was regeneration of the chorda tympani nerve and taste buds reappeared with innervation. In contrast, few taste buds were reinnervated in mice lacking normal *Bdnf* expression such that taste bud number remained low. In all genotypes, taste buds that were reinnervated were normal-sized, but non-innervated taste buds remained small and atrophic. On the side of the tongue contralateral to the nerve section, taste buds for some genotypes became larger and all taste buds remained innervated. Our findings suggest that BDNF is required for nerve regeneration following gustatory nerve section.

1. Introduction

The peripheral taste system is extremely plastic in adulthood: taste receptor cells die and are replaced (Beidler and Smallman, 1965), and must be newly innervated by nerve fibers. Thus, reinnervation occurs regularly in the normal taste system, even in the absence of nerve injury. This rich plasticity might explain why regeneration in the taste system after nerve injury is so robust (Cheal and Oakley, 1977; Reddaway et al., 2012; Shuler et al., 2004). When taste nerves are sectioned, taste buds are either completely lost or exhibit altered morphology (Fujimoto and Murray, 1970; Guagliardo and Hill, 2007; Oakley et al., 1990). Most taste buds, however, are regenerated and display normal morphology with taste nerve regeneration (Guth, 1957; St John et al., 1995), resulting in functional recovery (Cheal et al., 1977; McCluskey and Hill, 2002; Yasumatsu et al., 2003). Thus, gustatory nerve fibers are required to support normal taste bud number and morphology. In summary, the taste system is a highly plastic system with a close trophic relationship between taste buds and their innervating fibers.

Although regeneration in the taste system has been widely studied (Cain et al., 1996; Guth, 1957; Takeda et al., 2013), no factor required for gustatory nerve regeneration has yet been identified. We postulate that the neurotrophin BDNF might regulate taste nerve regeneration for several reasons. First, BDNF is required for innervation of taste buds during development (Hoshino et al., 2010; Lopez and Krimm, 2006; Ma et al., 2009; Nosrat et al., 1997; Sun and Oakley, 2002). Secondly, BDNF continues to be expressed in the adult taste system (Ganchrow et al., 2003a; Huang et al., 2015; Yee et al., 2003), where it is required to maintain normal levels of innervation (Meng et al., 2015). Thirdly, exogenous application of neurotrophins can enhance peripheral nerve regeneration (Fine et al., 2002; Funakoshi et al., 1998; Novikov et al., 1997; Sterne et al., 1997). Lastly, endogenous neurotrophins are required for motor neuron regeneration (Wilhelm et al., 2012; Zhang et al., 2000). While these findings point to a role for neurotrophins in sensory nerve regeneration, there is no direct evidence to support this hypothesis. The neurotrophin, nerve growth factor (NGF), is not required for sensory neuron regeneration (Diamond et al., 1992; Lankford et al., 2013), even though it is critical for peripheral innervation during development (Davis et al., 1997; Patel et al., 2000). Surprisingly, application of BDNF on the combined chorda tympani and lingual branch of the trigeminal nerves has no impact on nerve regeneration in the cat (Yates et al., 2004). Therefore, although there are

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good reasons to speculate that BDNF may be required for taste nerve regeneration, there are also reasons to think it may not be involved.

We sought to determine whether endogenous BDNF is required for regeneration of gustatory nerve fibers after unilateral chorda tympani nerve section. We report that following nerve section, BDNF expression remained high in the taste system, and BDNF was required for reinnervation of taste buds following unilateral gustatory nerve section.

2. Materials and methods

2.1. Animals

C57BL/6 J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Surgical groups underwent unilateral chorda tympani nerve section at 2 months of age. The geniculate ganglion and anterior tongue were dissected 2, 14, 30, or 60 days (all n = 6; half male and half female) after nerve section. Non-surgical control animals were age-matched with nerve-sectioned animals. *Bdnf*$^{LacZ/+}$ mice, in which the *BDNF* coding sequence at one allele is replaced by the *E. coli* galactosidase (*LacZ*) sequence, were used to determine the localization of BDNF expression (Jones et al., 1994; Yee et al., 2003) were obtained from Kevin Jones.

To globally deplete BDNF, mice expressing a tamoxifen-inducible CreERT2 recombinase under the control of the ubiquitin promoter (Jackson Laboratories, stock 007001) were bred with mice in which exon 5 of *Bdnf* was floxed (*Bdnf*$^{lox/lox}$, Jackson Laboratories, stock 004339). To efficiently manipulate *Bdnf* expression, one allele of *Bdnf* was deleted by crossing the resultant mice with *Bdnf*$^{+/-}$ mice (Jackson Laboratories, stock 002266). Therefore, the animals used in this study lacked a functional *Bdnf* allele, and *Bdnf* could be inducibly deleted from the other allele (CreER:*Bdnf*$^{lox/-}$). Three control genotypes were utilized for different comparisons. *Bdnf*$^{lox/+}$ mice (with tamoxifen) were used to exclude any potential effects of tamoxifen administration. CreER:*Bdnf*$^{lox/+}$ mice (without tamoxifen) were used to exclude the possibility of gene recombination in the absence of tamoxifen. Both of these mutants produce wild-type levels of *Bdnf* mRNA expression (Meng et al., 2015). *Bdnf*$^{lox/-}$ mice (with tamoxifen) were used to control for any effects of heterozygous *Bdnf* deletion. Non-surgical control mice for each genotype were littermates of the sectioned animals and received tamoxifen at the same time. Genotypes and manipulations are also shown in Table 1.

2.2. Tamoxifen administration

Tamoxifen (Sigma, St. Louis, MO, T5648, mixed with sunflower oil, 188 ng/g body weight) was administered once per day for 1 week by oral gavage. This dose has been previously used to effectively induce gene recombination in adult mice (McGraw et al., 2011; Meng et al., 2015; Ruzankina et al., 2007). Tamoxifen administration was initiated in all mice between post-natal days 60–70. Chorda tympani nerve section was performed 2 weeks after the end of tamoxifen administration. Mice were sacrificed 4 or 10 weeks after the final injection of tamoxifen (corresponding to 2 or 8 weeks after unilateral chorda tympani nerve section).

2.3. Unilateral chorda tympani nerve section

Mice were anesthetized with an intramuscular injection of dexmedetomadine hydrochloride (0.4 mg/kg body weight) followed by ketamine hydrochloride (40 mg/kg body weight) (Guagliardo and Hill, 2007). The surgical procedure was the same as used previously in rats (Hill and Phillips, 1994; Shuler et al., 2004). Mice were placed in a non-traumatic head restraint for ventral access to the chorda tympani nerve at the level of the neck. The chorda tympani nerve was located at its bifurcation from the lingual branch of the trigeminal nerve and was sectioned without damaging the trigeminal nerve. The surgical site was sutured and mice were allowed to recover on a water-circulating heating pad before being returned to their home cage. Atipamezole hydrochloride (2 mg/kg body weight) was injected intramuscularly immediately after surgery to counteract the effects of dexmedetomadine and thereby decrease recovery time from the anesthetic.

2.4. RT-PCR

Mice were sacrificed by an overdose of Avertin (2.5% tribromoethanol; T48402, Sigma-Aldrich, St. Louis, MO). The anterior tongue rostral to the circumvallate papilla was removed and rinsed with 0.1 M phosphate buffered saline (PBS) (pH 7.4) and then cut in half evenly under a microscope. To isolate the entire lingual epithelium, tongue tissue containing fungiform papillae was incubated in sterile dispase I-solution (BD Biosciences, San Jose, CA) for 30–40 min. After incubation, epithelial sheets were peeled from the underlying lamina propria. The lingual epithelium from each mouse was stored in RNAlater

Table 1
Animal genotypes, manipulations and tissues analyzed.

Genotype/number of animals	Tamoxifen injection	Surgery	Time post-tamoxifen/post-surgery	Tissues analyzed	Type of analysis
Wild type n = 28	None	Unilateral CT section	2, 14, 30, 60 days	Tongue epithelium/geniculate ganglion	RT-PCR
Wild type n = 4	None	Unilateral CT section	14 days	Taste buds/geniculate ganglion	RT-PCR
Bdnf$^{lacZ/+}$ n = 3	None	None		Tongue	βGal histochemistry
Bdnf$^{lox/+}$ n = 3	Yes	None	14 days	Tongue epithelium/geniculate ganglion	RT-PCR
CreER:*Bdnf*$^{lox/+}$ n = 3	None	None	14 days	Tongue epithelium/geniculate ganglion	RT-PCR
Bdnf$^{lox/}$ n = 3^{-}	Yes	None	14 days	Tongue epithelium/geniculate ganglion	RT-PCR
CreER:*Bdnf*$^{lox/-}$ n = 3	Yes	None	14 days	Tongue epithelium/geniculate ganglion	RT-PCR
Bdnf$^{lox/+}$ n = 3	Yes	Unilateral CT section	28 days/14 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/+}$ n = 3	None	Unilateral CT section	28 days/14 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/-}$ n = 3	Yes	Unilateral CT section	28 days/14 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/-}$ n = 3	Yes	Unilateral CT section	28 days/14 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/+}$ n = 3	Yes	None	28 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/+}$ n = 3	None	None	28 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/-}$ n = 3	Yes	None	28 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/-}$ n = 3	Yes	None	28 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/+}$ n = 3	Yes	Unilateral CT section	70 days/56 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/+}$ n = 3	None	Unilateral CT section	70 days/56 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/-}$ n = 3	Yes	Unilateral CT section	70 days/56 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/-}$ n = 3	Yes	Unilateral CT section	70 days/56 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/+}$ n = 4	Yes	None	70 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/+}$ n = 4	None	None	70 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
Bdnf$^{lox/-}$ n = 4	Yes	None	70 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number
CreER:*Bdnf*$^{lox/-}$ n = 4	Yes	None	70 days	Tongue/geniculate ganglion	Taste bud anatomy, neuron number

stabilization solution (Ambion, Austin, TX), and geniculate ganglia were stored in RNA isolation reagent (Qiagen, Chatsworth, CA) at −80 °C until RNA extraction. For laser capture microdissection, tissues were frozen in RNase-free containers for sectioning.

For laser capture microdissection, mice underwent unilateral nerve section and 14 days later tissue was isolated (n = 3). Geniculate ganglia and tongues halves (cut and uncut side) were cut into 10-μm thick sections on a cryostat and mounted on slides. Sections were dehydrated using serial concentrations of alcohol and xylene. After dehydration, tissues were placed on a laser capture microdissection microscope (Arcturus, ThermoFisher Scientific). Geniculate ganglia and taste buds were identified, laser-captured, and then collected in tubes containing total RNA isolation reagent (Qiagen). For geniculate ganglia, the entire region containing neurons for each section was captured. For each side of the tongue, all taste buds that could be identified without staining in each section were captured (25–30 taste buds/sample).

Expression levels of mRNA in the tongue epithelium, geniculate ganglia, and taste buds were measured using RT-PCR. Total RNA from tissues was extracted using an RNeasy Micro Kit or RNeasy Mini Kit (Qiagen). DNase I was applied to eliminate traces of DNA during the procedure. After extraction, RNA was analyzed using RNA 6000 Pico/Nano Chip Kits in a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), and RNA Integrity Number (RIN) and 28S:18S ratio were used to estimate RNA quality. Only RNA samples with RIN > 8.0 were used in this study. Reverse transcription was performed using 200 U Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) and 50-ng random hexamers (Invitrogen) in a 25-μL reaction volume containing first strand buffer (Invitrogen), 0.5 mM dNTPs, and 40 U RNase inhibitor. To control for differences in the amount of RNA isolated from different groups, the same amount of RNA was used from each geniculate ganglion (3 ng) and lingual epithelium (50 ng) sample for cDNA synthesis.

RT-PCR was performed using the ABI PRISM/7900HT sequence detection system (Applied Biosystems, Foster City, CA), the TaqMan Universal PCR Kit (Applied Biosystems), and oligonucleotide primer/probe sets, which were designed from sequences available in the GenBank database (Table 2) using Beacon Designer software (Premier Biosoft International, Atlanta, GA). Where possible, primers were selected to span an intron to avoid amplification of genomic DNA contamination. TaqMan probes were labeled at the 5′-end with a fluorescent reporter dye (fluorescein, FAM) and at the 3′-end with a quencher dye (carboxytetramethylrhodamine, TAMRA). RT-PCR reactions were conducted using 10-μL total volumes with Master Mix 720/200 nM primer/probe sets (TaqMan PCR Kit). To normalize cDNA loading, all samples were run in parallel with the housekeeping genes, 18S ribosomal RNA, and phosphate GAPDH. Each assay was performed in triplicate. For quantification, the comparative $2^{-\Delta\Delta CT}$ method was employed to determine the expression changes of each target gene for each animal relative to housekeeping genes (Livak and Schmittgen, 2001).

2.5. β-galactosidase histochemistry

Mice were sacrificed by an overdose of Avertin (4 mg/kg body weight) and perfused and tissues immersion-fixed in ice-cold 0.5% glutaraldehyde in PBS/$MgCl_2$ solution (2 mM $MgCl_2$ in PBS) for 1–2 h at 4 °C. Fixative was removed by multiple washes in ice-cold PBS/$MgCl_2$. Tissues were frozen on dry ice in optimal cutting temperature (OCT) embedding medium and stored at −80 °C. Tissue sections (40 μm) were mounted on SuperFrost Plus slides and allowed to air-dry for 1 h at 4 °C. β-galactosidase histochemistry was performed on sectioned tissue. Tissue sections were washed in ice-cold PBS/$MgCl_2$ to thoroughly remove OCT and then immersed in X-gal staining solution (InvivoGen, San Diego, CA, 0.02% Igepal, 0.01% sodium deoxycholate,

Table 2
Sequences of primer pairs and probes used for real-time RT-PCR.

Gene GenBack accession #		Sequence 5′-3′	Size (bp)
Bdnf (X55573)			110
	Forward primer	TGCAGGGGCATAGACAAAAGG	
	Reverse primer	CTTATGAATCGCCAGCCAATTCTC	
	Taqman probe	ACTGGAACTCGCAATGCCGAACTACCCA	
Ntf4 (NM_198190)			95
	Forward primer	AGCGTTGCCTAGGAATACAGC	
	Reverse primer	GGTCATGTTGGATGGGAGGTATC	
	Taqman probe	TGAGCAGTGAACCCGACCACCCAGG	
Ntf3 (NM-001164034)			94
	Forward primer	CAGAACATAAGAGTCACCGGAA	
	Reverse primer	TGTCCCCGAATGTCAATGG	
	Taqman probe	CACCCACAGGCTCTCACTGTC	
TrkB (X17647)			86
	Forward primer	AAGGACTTTCATCGGGAAGCTG	
	Reverse primer	TCGCCCTCCACACAGACAC	
	Taqman probe	CCAACCTCCAGCACGAGCACATTGTCAA	
P2rx3 (MN-145526)			113
	Forward primer	TTTCCCCTGGCTACAACTTC	
	Reverse primer	CCCGTATACCAGCACATCAAAG	
	Taqman probe	AGATGGAGAATGGCAGCGAGTACCG	
18S rRNA (X00686)			76
	Forward primer	CAGGATTGACAGATTGATAGCTCTTTC	
	Reverse primer	ATCGCTCCACCAACTAAGAACG	
	Taqman probe	CCATGCACCACCACCCACGGAATCG	
GAPDH (NM_008084)			130
	Forward primer	AATGTGTCCGTCGTGGATCTG	
	Reverse primer	CAACCTGGTCCTCAGTGTAGC	
	Taqman probe	CGTGCCGCCTGGAGAAACCTGCC	
NefL (NM_010910)			67
	Reverse primer	AGCGGGAAGGCTCAGAGTG	
	Taqman probe	CGAGCTGTTGGTGCTGCGCCAGA	
Krt8 (NM_031170)			130
	Forward primer	TCTTCTGATGTCGTGTCCAAGTG	
	Reverse primer	GATCCTCGGACGGGTCTCTAG	
	Taqman probe	CCACTGAAGTCCTTGCCAGCCTGAGC	

5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mg/mL X-gal in PBS/$MgCl_2$) for 2–6 h in a 37 °C incubator. Slides were washed with PBS and allowed to dry overnight in a 37 °C heater. Sections were mounted using Glycergel mounting medium (Dako North America, Carpinteria, CA) and imaged using a bright field camera.

2.6. Immunohistochemistry

Mice were sacrificed by an overdose of avertin (4 mg/kg body weight), transcardially perfused with 4% paraformaldehyde (PFA), and post-fixed in PFA for 2 h or immersion-fixed in 4% PFA overnight. Geniculate ganglia were dissected under a microscope. Tissues were then rinsed with PBS and incubated in 30% sucrose at 4 °C overnight. Tissues were frozen the next day in OCT and stored at −80 °C until sectioning on a cryostat. Serial sagittal sections of the tongue (20 μm) and geniculate ganglion (50 μm) were mounted on glass slides.

To visualize taste buds and innervation in serial sections, slides containing tongue tissue were first dried on a slide warmer (37 °C) overnight. The next day, slides were rehydrated, placed in citric acid buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0), heated to 98–100 °C for approximately 15 min in a boiling water bath, and allowed to cool for 20 min at room temperature for antigen retrieval. Slides were washed with PBS (pH 7.4) and incubated overnight at room temperature with an anti-cytokeratin 8 antibody in PBS (supernatant, 1:50, Developmental Studies Hybridoma Bank, Troma-1-s, Iowa City, IA). After incubation with the primary antibody, slides were rinsed in PBS and incubated for 1.5 h with an Alexa 488 anti-rat secondary antibody (1:500, Invitrogen). After washing in PBS, slides were incubated for 2 h in blocking solution (5% normal goat serum and 2% Triton X-100 in PBS), followed by incubation with rabbit anti-P2X3 (1:500, Millipore, Billerica, MA, AB5895) and mouse anti-TUJ1 (1:500, Covance, San Diego, CA, MMS-435P) antibodies in blocking solution at room temperature overnight. After overnight incubation, slides were rinsed with PBS and incubated for 1.5 h at room temperature with Alexa 555 anti-rabbit (1:500, Invitrogen) and Alexa 647 anti-mouse (1:500, Invitrogen) secondary antibodies in blocking solution. Slides were then rinsed with PBS and stained with DAPI (2 μL in 50 mL double-distilled water, Life Technologies, Foster City, CA) for 15 min. After rinsing with PBS, slides were mounted with fluoromount-G (SouthernBiotech, Birmingham, AL).

2.7. Quantification of taste bud number, size, and innervation

Taste buds were counted in 20-μm serial sagittal sections of the tongue, which included everything rostral of the circumvallate papillae using a Leica DMLB microscope (W. Nuhsbaum, Inc., McHenry, IL). The entire taste bud was followed through each section, such that taste buds were counted only once. In addition, each taste bud was examined for the presence or absence of P2X3-positive or TU11-positive innervation, in order to determine the percentage of taste bud that were innervated.

To measure the volume of innervation and taste cell number per taste bud, individual taste buds (6/tongue half) were imaged for analysis using an Olympus Fluoview, FV1000 laser scanning confocal microscope (LSI3-FV1000-Inverted, B&B Microscopes Limited, Pittsburgh, PA). Because taste buds are larger on the back of the tongue than at the tip (Biggs et al., 2016), taste buds were chosen from the front one-third of the fungiform field in order to reduce anterior-posterior variation in taste bud size. During both image acquisition and analysis, the investigator was blind to each specimen's genotype. For each optical image, the signals from four channels were collected separately using single-wavelength excitation, and then merged to produce a composite image. Serial optical sections were captured every 1 μm in labeled whole taste buds at 60× magnification with the zoom set at 3.5. As a single taste bud was typically identified in two or three sections (i.e., spanning 36–60 μm), all sections containing a given taste bud were captured, such that the entire taste bud could be analyzed. Each physical section contained 15–20 optical sections. Each optical section was traced (Meng et al., 2015), and the traced area was measured using Neurolucida imaging software (MicroBrightField, Bioscience, Williston, VA). The area of each optical section was summed across all physical sections to yield taste bud volume. The volume of anti-P2X3 and anti-TUJ1 immunostaining in taste buds was measured using MBF ImageJ software (ImageJ 1.47) (Jensen, 2013), which sets an unbiased threshold automatically, and pixels were analyzed for every section. The number of labeled pixels inside a taste bud for each optical section was summed to obtain the total volume occupied by nerve fibers for each taste bud.

Taste cell number was determined by counting DAPI staining within cytokeratin 8-positive taste buds. DAPI-labeled nuclei were followed through optical sections, such that each nucleus was counted only once. As each taste bud appeared in more than one physical section, the number of cells was summed across all physical sections. A small number of cells may have been counted twice in cases of split nuclei.

2.8. Stereology

Stereological methods were used to count the total number of geniculate ganglion neurons. Fixed ganglia were serially sectioned (50 μm), mounted on slides, and stained with cresyl violet for 15 min. To maintain section thickness, sections were not dehydrated and were mounted in Dako Glycergel mounting medium (Dako North America) for stereological quantification. Using StereoInvestigator software (MBF Bioscience), a blind investigator traced a contour around the geniculate ganglion at 20× magnification. Every section containing the ganglion was traced, and at least four sections were quantified. Within each traced contour, the software randomly determines the placement of the counting frames. The depth of the counting frame was equal to the minimal thickness of the section minus a 10-μm guard zone (i.e., 5 μm from the top and bottom of the section). Geniculate ganglion neurons were counted at 100× magnification in each counting frame (15 μm^2). Neurons were counted only when they first came into focus (cell top), such that each cell was counted only once. Based on these measurements, total cell number in each ganglion was estimated for the entire volume of the ganglion using the optical fractionator probe (MBF Bioscience).

2.9. Statistical analysis

All measurements in a given group were determined to have equal variance (Levene's test for homogeneity of variance, $p > 0.05$). For statistical testing of *Bdnf* mRNA levels in wild-type mice following nerve section, one-way ANOVAs were used. For testing *Bdnf* mRNA levels in transgenic mice, a two-way ANOVA was used. For taste bud numbers, geniculate neuron numbers, taste bud volumes, nerve innervation, taste cell numbers, taste bud numbers, and ganglion cell numbers, three-way ANOVAs were used (four genotypes by 2 treatments (chorda tympani sectioned vs unsectioned)) at two times after treatment (2 and 8 weeks). For analysis of individual taste buds, the mean value from each mouse represents six different taste buds. After overall significance was determined, Tukey post-hoc tests were used to identify significant differences in pairwise comparisons when differences were found across genotype of $p < 0.05$. The mean value of each group represents 3–4 mice. Statistical significance was set at $p < 0.05$ for all comparisons; actual p-values are provided except when they were below the lowest reporting limit of the software ($p < 0.001$).

3. Results

3.1. Chorda tympani nerve section does not reduce BDNF expression in the geniculate ganglion or lingual epithelium

Neurotrophins are postulated to promote nerve regeneration after nerve section. In the peripheral taste system, BDNF is primarily

expressed in taste buds (Yee et al., 2003), but is also expressed in the geniculate ganglion (Harlow et al., 2011; Huang and Krimm, 2010). Because taste bud number and size decrease following section of the chorda tympani nerve (Guagliardo and Hill, 2007), resulting in a loss of BDNF in taste buds (Ganchrow et al., 2003b), there may be little BDNF remaining in the taste bud. Thus, if BDNF expression is diminished in taste buds or geniculate ganglia after nerve section, it is unlikely to play a role in chorda tympani nerve regeneration.

To determine if this was the case, *Bdnf* expression in the geniculate ganglion was measured 2, 14, 30, or 60 days (n = 5–6/group) after unilateral chorda tympani nerve section. Expression of the neuronal marker, neurofilament, was also examined to ensure that an equal number of neurons were isolated from each geniculate ganglion. Expression of neurofilament was not significantly different between all groups ($F_{(4,13)} = 0.20$ p = 0.93), indicating that a relatively equal number of neurons was isolated from each geniculate ganglion. *Bdnf* expression did not significantly change in the geniculate ganglion after unilateral chorda tympani nerve section (Fig. 1A, $F_{(4,15)} = 0.88$, p = 0.49). We also measured expression of other neurotrophins, capable of binding the same receptor as BDNF, in the geniculate ganglion 14 days after nerve section using qRT-PCR. Neurotrophin 3 (*Ntf3*) expression did not change significantly (relative mRNA levels: non-surgical controls = 1 ± 0.08, intact side = 1.20 ± 0.08, sectioned side = 0.94 ± 0.04, $F_{(2,6)} = 3.77$ p = 0.09). Neurotrophin 4 (*Ntf4*) was not detectable in the adult geniculate ganglion. *TrkB* (BDNF receptor) expression remained high in the adult geniculate ganglion and was unchanged 14 days after nerve section ($F_{(2,6)} = 3.17$ p = 0.11). These results suggest that the expression of neurotrophins (*Bdnf*, *Ntf3*, and *Ntf4*) and the primary BDNF receptor (*TrkB*) in the geniculate ganglion was not affected by unilateral chorda tympani nerve section.

Bdnf expression was also examined in the lingual epithelium following nerve section. *Bdnf* expression did not change in the lingual epithelium on the sectioned side when measured 2, 14, 30, or 60 days after nerve section ($F_{(4,23)} = 2.79$ p = 0.08; Fig. 1B). Consistent with observations of taste bud loss, expression of the taste bud-specific protein, cytokeratin 8, decreased after nerve section ($F_{(4,23)} = 7.047$ p = 0.001). Specifically keratin 8 was reduced 14 (p = 0.005), 30 (p = 0.002), and 60 days (p = 0.005) following nerve section (Fig. 1C). Therefore, while there was a loss of taste buds in the lingual epithelium, *Bdnf* expression was not reduced.

One possible explanation for why *Bdnf* expression remains normal despite the loss of taste buds/cells is that the remaining taste cells compensate by increasing *Bdnf* expression. To test this hypothesis, we individually isolated the remaining taste buds using laser capture microdissection 14 days following nerve section, and *Bdnf* expression was analyzed. Expression of *Bdnf* (non-surgical controls = 1 ± 0.15 (mean ± SEM), nerve-sectioned = 0.22 ± 0.03; $T = 5.08$, p = 0.007) and cytokeratin 8 (non-surgical controls = 1 ± 0.08, nerve-sectioned = 0.11 ± 0.02; $T = 10.33$ p < 0.001) in the remaining taste buds decreased in the sectioned side compared with taste buds from non-surgical controls. These decreases are likely due to a reduction in the size of remaining taste buds (Guagliardo and Hill, 2007). When *Bdnf* expression is calculated relative to cytokeratin 8 expression to account for the reduction in taste bud size, relative *Bdnf* expression is elevated 14 days after nerve section (2.5 ± 0.35) compared with non-sectioned controls (1 ± 0.25; $T = 3.49$, p = 0.03). This finding suggests that there is an increase in *Bdnf* expression in the remaining taste cells following nerve section.

Another potential explanation for why *Bdnf* expression remains elevated in the lingual epithelium after nerve section is that *Bdnf*

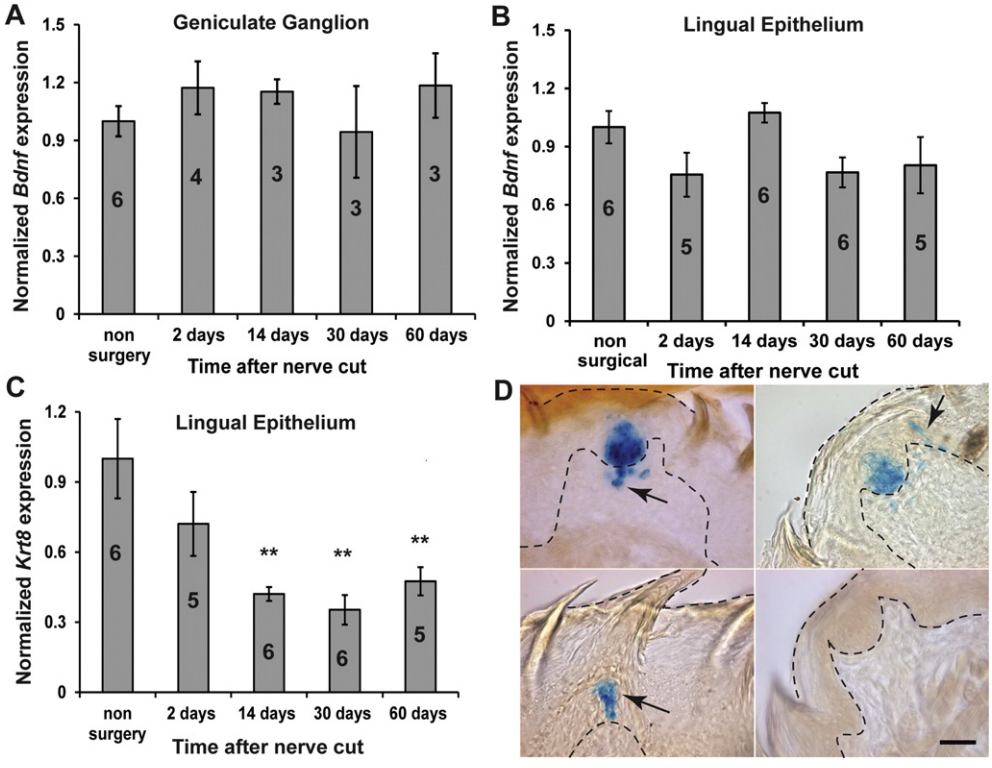

Fig. 1. *Bdnf* continues to be expressed in the geniculate ganglion and lingual epithelium after chorda tympani nerve section, while expression of the taste bud marker, cytokeratin 8 (*Krt8*), is decreased. *Bdnf* mRNA expression levels (normalized to non-surgical controls) as determined by qRT-PCR did not change 2, 14, 30, or 60 days after nerve section in the geniculate ganglion (A) and the lingual epithelium (B). In contrast, *Krt8* expression levels (normalized to non-surgical controls) in the epithelium decreased 14, 30 and 60 days after nerve section (C). Numbers in each bar are the number of animals contributing to each data point. These findings are consistent with loss and recovery of taste buds following nerve section, with no loss of *Bdnf* expression, possibly because *Bdnf* expression is not limited to taste buds. (D) We observed *Bdnf* expression in both taste and non-taste cells in the tongue. Dashed lines illustrate the border between the laminia propria and the epithelium and also the tongue surface. In a fungiform papilla from a $Bdnf^{Lacz/+}$ mouse, the β-galactosidase reaction product can be seen in the taste bud and in the lamina propria ventral to the taste bud (top left arrow) and immediately adjacent to the taste bud (top right arrow). Occasionally, the β-galactosidase reaction product was observed in the filiform epithelium of $Bdnf^{Lacz/+}$ mice, but never in wild-type mice (D, bottom right). **p < 0.01.

expression is not limited to taste buds. To test this hypothesis, non-taste regions of the lingual epithelium were isolated using laser capture microdissection and *Bdnf* expression was analyzed. In wild-type mice, the lamina propria and filiform papillae expressed *Bdnf* with no change in expression levels 14 days after nerve section (laminia propria: non-surgical controls = 1 ± 0.01, nerve-sectioned = 0.81 ± 0.01; $T = 2.59$, $p = 0.17$; filiform papillae: non-surgical controls = 1 ± 0.01, nerve-sectioned = 1.20 ± 0.01; $T = -3.73$, $p = 0.07$). β-galactosidase staining in $Bdnf^{lacZ/+}$ mice confirmed that *Bdnf* is expressed in both taste and non-taste regions (Fig. 1D).

We conclude that *Bdnf* expression remains unchanged in the lingual epithelium following unilateral chorda tympani nerve section for two reasons. First, the remaining taste cells increase *Bdnf* expression presumably to compensate for the loss of *Bdnf*-expressing taste cells, while non-taste bud *Bdnf* expression also contributes to total lingual *Bdnf* expression. This finding is consistent with earlier findings that BDNF is somewhat reduced in fungiform taste buds but not eliminated following chorda tympani nerve section (Ganchrow et al., 2003b). Others have also found that *Bdnf* expression increases in sectioned taste nerves (Yee et al., 2005), likely due to increased expression by the Schwann cells. Thus, *Bdnf* expression in the remaining ganglion neurons, taste cells, lingual epithelium, and the chorda tympani nerve following nerve section may support nerve regeneration.

3.2. Bdnf gene deletion prevents or delays recovery of taste bud number following chorda tympani nerve section

Because *Bdnf* continues to be expressed in the geniculate ganglion, lingual epithelium, and remaining taste cells after unilateral chorda tympani nerve section, we postulated that BDNF supports nerve regeneration after injury. To test this hypothesis, *Bdnf* was deleted from adult mice using a tamoxifen-inducible CreERT2 (CreER) construct under the control of the ubiquitin promoter. The effectiveness of *Bdnf*-mediated gene recombination in these mice has been extensively studied (Meng et al., 2015). Tamoxifen was administered 2 weeks prior to unilateral chorda tympani nerve section (Fig. 2A), by which time *Bdnf* mRNA expression levels in the lingual epithelium were reduced to 4% of normal levels ($Bdnf^{lox/+}$ 1.0 ± 0.2, CreER:$Bdnf^{lox/+}$ 1.1 ± 0.2, $Bdnf^{lox/-}$ 0.87 ± 0.05, CreER:$Bdnf^{lox/-}$ 0.04 ± 0.01, ($F_{(1,4)} = 7.71$ $p = 0.005$)). Maintenance of fungiform taste buds is dependent upon innervation, such that 68% of taste buds are lost following nerve section (Oakley et al., 1993; Guagliardo and Hill, 2007). These taste buds are regenerated when innervation is restored (Guagliardo and Hill, 2007). We therefore measured taste bud number in this series of experiments.

Total taste bud number on the sectioned side of the tongue was quantified in serial sections following tamoxifen injection 2 or 8 weeks following nerve section (Fig. 2B,C). Consistent with previous reports (Guagliardo et al., 2009; Whitehead et al., 1987), some taste buds were still present 2 weeks after nerve section in all four genotypes and continued to be present 8 weeks after nerve section (Fig. 2D). Taste bud number was reduced by both nerve sectioning ($F_{(1,38)} = 313$, $p < 0.001$) and genotype ($F_{(3,38)} = 3.62$, $p = 0.02$). Specifically, taste bud number decreased by almost half in all four genotypes 2 weeks after nerve section ($p < 0.001$ for all genotypes), Fig. 2D), indicating that *Bdnf* deletion did not affect taste bud loss following nerve section. Taste bud number was still reduced 8 weeks after nerve section ($p < 0.001$, Fig. 2D), suggesting that regeneration was not yet (or would never be) complete. Eight weeks after nerve section, taste bud number increased by 40% in control ($Bdnf^{lox/+}$ $p = 0.006$, CreER:$Bdnf^{lox/+}$, $p = 0.001$) and heterozygous *Bdnf* mutant mice ($Bdnf^{lox/-}$ $p = 0.01$) compared with 2 weeks after nerve section, demonstrating substantial recovery in mice with BDNF. In contrast, mice with *Bdnf* deletion (CreER:$Bdnf^{lox/-}$) did not show a robust recovery in taste bud number ($p = 0.918$, Fig. 1D). Our results show that *Bdnf* deletion does not affect taste bud loss shortly after unilateral chorda tympani nerve section, but instead delays or prevents recovery of taste bud number much later (8 weeks) after injury.

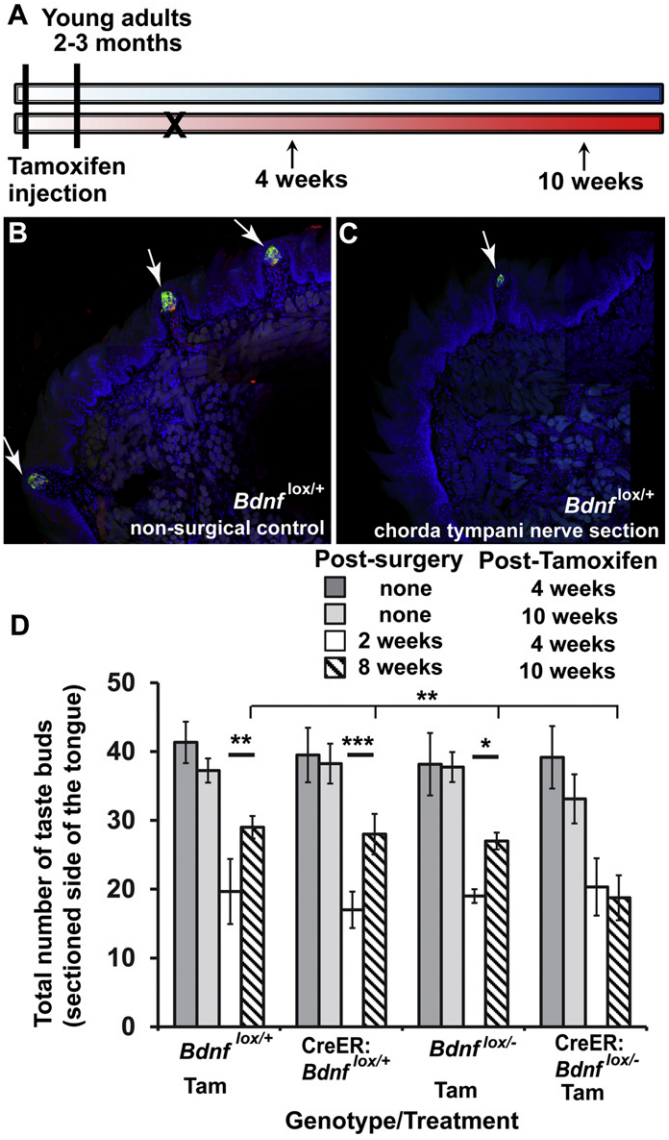

Fig. 2. *Bdnf* deletion did not affect taste bud loss 2 weeks after chorda tympani nerve section, but slowed or prevented recovery of taste bud number 8 weeks after nerve section. Experimental timeline (A) for non-surgical controls (blue) and chorda tympani nerve-sectioned mice (red). Tissues were collected 4 and 10 weeks after tamoxifen (Tam) administration, corresponding to 2 and 8 weeks after chorda tympani nerve section. The total number of taste buds (arrows) were quantified in serial sections on the sectioned side of the tongue in four genotypes following tamoxifen injection, coupled with either no surgery (B, $Bdnf^{lox/+}$) or chorda tympani nerve section (C, $Bdnf^{lox/+}$, 2 weeks after section). Two weeks after nerve section (D), the number of taste buds decreased by half in mice of all four genotypes ($Bdnf^{lox/+}$, CreER:$Bdnf^{lox/+}$, $Bdnf^{lox/-}$, CreER:$Bdnf^{lox/-}$, all n = 3) compared with non-surgical controls 4 (n = 3) and 10 weeks (n = 4) after tamoxifen treatment. Eight weeks after nerve section, taste buds returned in $Bdnf^{lox/+}$, CreER:$Bdnf^{lox/+}$, and $Bdnf^{lox/-}$ mice, but not in CreER:$Bdnf^{lox/-}$ mice (all n = 3). There was no significant difference in *Bdnf* expression level between the three control genotypes. These data demonstrate that normal *Bdnf* levels are required for taste bud number to recover following nerve section. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3. Bdnf deletion prevents or delays taste bud reinnervation

Since taste bud number did not recover 8 weeks after chorda tympani nerve section in mice with reduced *Bdnf* expression, and because taste bud maintenance is dependent upon innervation (Oakley et al., 1993; Guagliardo and Hill, 2007), it is likely that reinnervation was also impaired following nerve section. To test this hypothesis, we

analyzed nerve fibers in cytokeratin 8-positive taste buds (green) using two markers, P2X3 (red) and TUJ1 (blue), a neuron-specific beta III tubulin (Fig. 3). P2X3 is a purinergic receptor required for neural responses to taste stimuli (Eddy et al., 2009; Finger et al., 2005; Murata et al., 2010; Vandenbeuch et al., 2015) and is primarily found in taste fibers in the tongue (Finger et al., 2005; Ishida et al., 2009). TUJ1 is present in all nerve fibers.

Two weeks after nerve section, there were no P2X3-positive fibers in all four genotypes, confirming previous studies reporting that P2X3 primarily labels taste fibers (Ishida et al., 2009). There were a few TUJ1-positive fibers in taste buds in all four genotypes, which may have come from the trigeminal nerve (Fig. 3A). Eight weeks after nerve section, most taste buds in control genotypes were reinnervated by P2X3-positive nerve fibers (Fig. 3B). In contrast, few taste buds were reinnervated by P2X3-positive fibers in CreER:*Bdnf*$^{lox/-}$ mice in which *Bdnf* was deleted. These taste buds also appeared to have fewer TUJ1-positive fibers compared with the other three genotypes (Fig. 3B).

Because taste bud number is affected by chorda tympani nerve section, we quantified the percentage of remaining taste buds that were innervated from serial sections of cut side of the tongue to examine

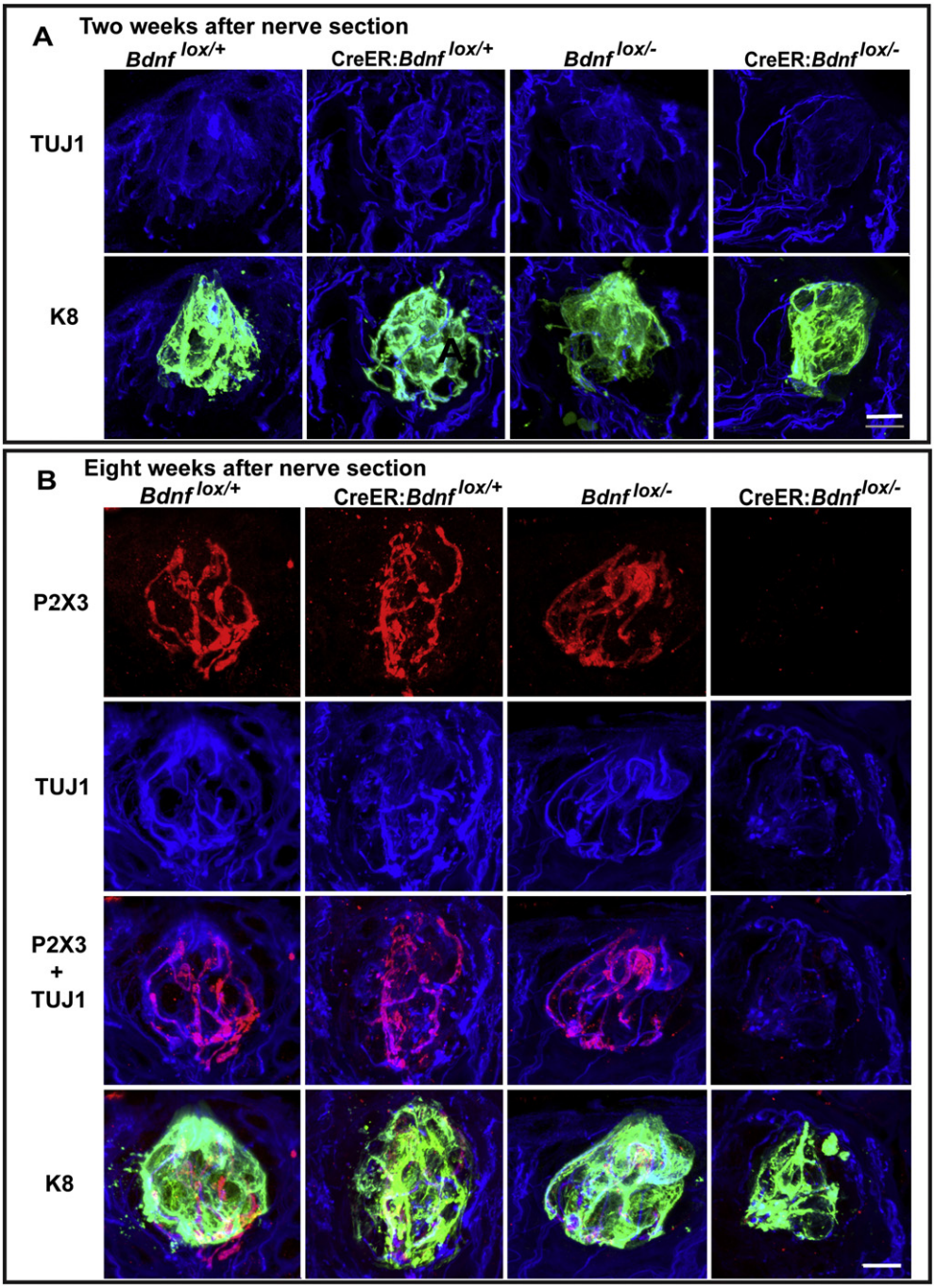

Fig. 3. Remaining taste buds lost most of their innervation 2 weeks after chorda tympani nerve section (A). For all genotypes, 2 weeks after chorda tympani nerve section, some TUJ1-positive fibers (blue), but no P2X3-positive fibers, were present in the remaining taste buds identified by staining with cytokeratin 8 (K8, green). There was no obvious difference among the four groups in the amount of remaining TUJ1-positive innervation following nerve section. Eight weeks after nerve section, both P2X3- and TUJ1-labeled nerve fibers were present in many taste buds of control genotypes (B), indicating that reinnervation had occurred. Few taste buds in *Bdnf*-deleted mice (CreER:*Bdnf*$^{lox/-}$), however, had P2X3-positive innervation and TUJ1-positive innervation also appears to have diminished, indicating that reinnervation was prevented or delayed by *Bdnf* deletion. Scale bar = 10 μm and applies to all panels.

reinnervation independent of taste bud loss. The number of taste buds innervated by P2X3-positive fibers was reduced by nerve sectioning ($F_{(1,36)} = 3489$, $p < 0.001$) in a genotype ($F_{(3,36)} = 54$, $p < 0.001$) and in a time-dependent manner ($F_{(1,36)} = 484$, $p < 0.001$). Specifically, two weeks after nerve section, no taste buds had P2X3-positive innervation, regardless of genotype or tamoxifen treatment, while all taste buds in non-surgical controls contained P2X3-positive fibers (Fig. 4A). This finding is consistent with previous studies demonstrating a complete loss of P2X3-positive innervation to the tongue following chorda tympani nerve section (Ishida et al., 2009). Eight weeks after nerve section, reinnervation of the taste bud was not complete, even though *Bdnf* expression levels were normal. Specifically, mice in all four genotypes still had fewer taste buds containing P2X3-positive fibers ($p < 0.001$ for all genotypes, Fig. 4A) compared with non-surgical controls. Nearly 70% of taste buds, however, eventually exhibited P2X3-positive fibers in mice expressing normal *Bdnf* expression ($p = 0.001$ for three control genotypes, Fig. 4A). In contrast, only 7.8% of the taste buds in mice lacking *Bdnf* regained P2X3-positive fibers, which was not a significant increase compared with 2 weeks after nerve section (CreER:$Bdnf^{lox/-}$ $p = 0.99$, Fig. 4A). Thus, substantially fewer taste buds regained P2X3-positive innervation when BDNF was reduced (CreER:$Bdnf^{lox/-}$ $p < 0.001$, Fig. 4A) compared with the other genotypes. Most taste buds were not reinnervated by P2X3-postive fibers in mice lacking BDNF.

We then analyzed the volume of P2X3-positive fibers in individual taste buds that were successfully reinnervated to determine whether reinnervated taste buds received normal levels of innervation. Taste buds are normally densely packed with innervation, such that individual fibers cannot be traced when all the innervation to the taste bud is labeled. Thus, the few gustatory studies attempting to quantify all innervation have measured the volume of P2X-positive pixels with the taste bud (Castillo et al., 2014; Huang et al., 2015; Meng et al., 2015). P2X3-positive innervation of individual taste buds was reduced

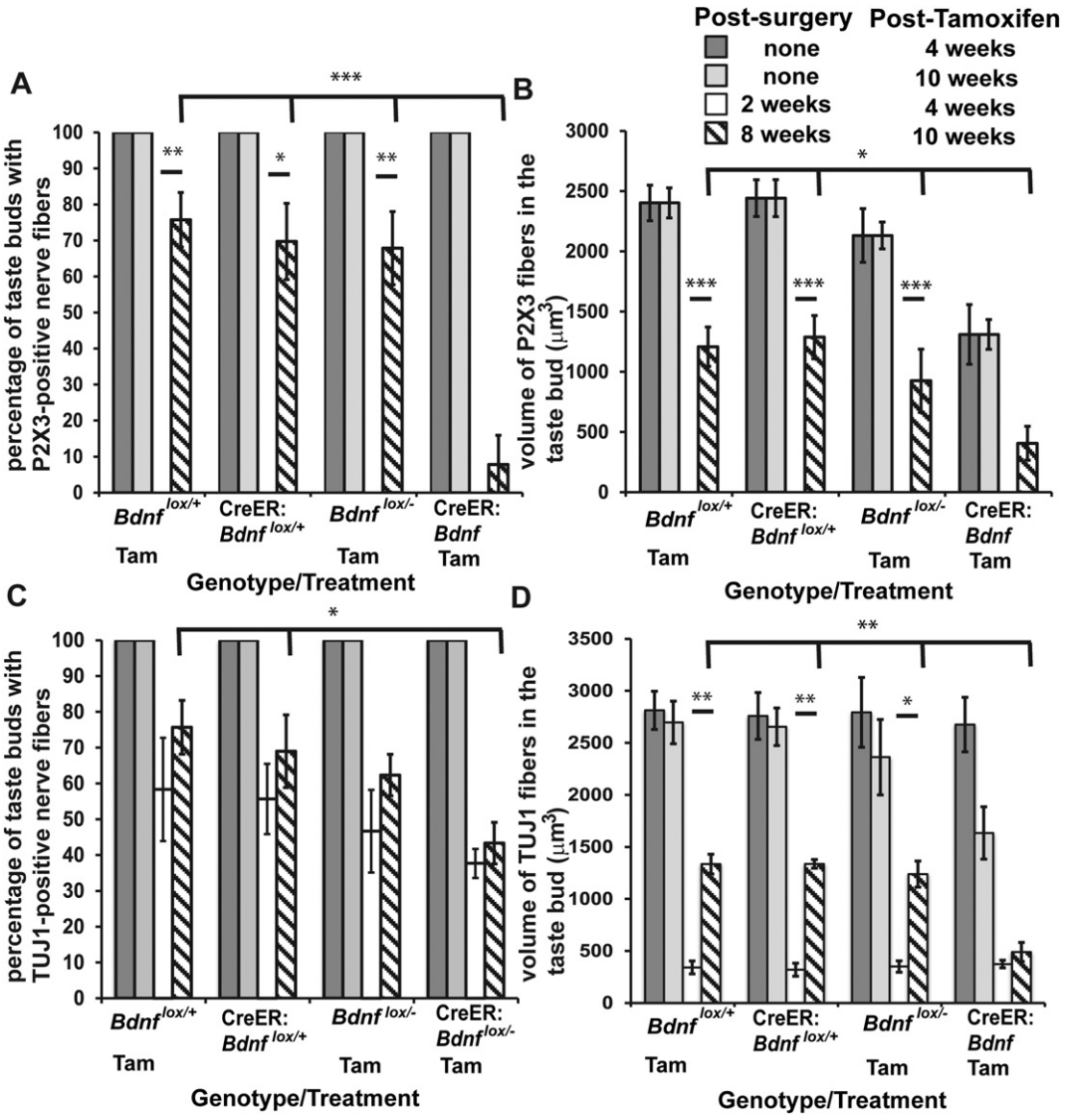

Fig. 4. *Bdnf* deletion prevented taste bud reinnervation after chorda tympani nerve section. In non-surgical controls (4 weeks post-tamoxifen, n = 3 each genotype; 10 weeks post-tamoxifen, n = 4 each genotype), all taste buds contained P2X3-positive fibers (A). Two weeks after section, taste buds in all genotypes lost P2X3-positive fibers (n = 3 each genotype). Eight weeks after section, the percentage of taste buds reinnervated by P2X3-labeled nerve fibers increased in mice expressing *Bdnf* ($Bdnf^{lox/+}$, CreER:$Bdnf^{lox/+}$, $Bdnf^{lox/-}$, all n = 3), but few taste buds regained P2X3-positive innervation when *Bdnf* was deleted (CreER:$Bdnf^{lox/-}$, n = 3) (A). Furthermore, the volume of P2X3-positive fibers was much lower in the few taste buds that regained innervation in CreER:$Bdnf^{lox/-}$ mice (B, n = 4) compared with taste buds from the three other genotypes. There was no difference in P2X3 labeling among the three control genotypes (B). As with P2X3-positive fibers, all taste buds contained TUJ1-positive fibers in the absence of injury (C, $Bdnf^{lox/+}$ n = 3, CreER:$Bdnf^{lox/+}$ n = 3, $Bdnf^{lox/-}$ n = 3, CreER:$Bdnf^{ox/-}$ n = 4).Two weeks after nerve section, only 50% of the taste buds contained TUJ1-positive fibers in mice of all four genotypes (n = 3 each genotype). Eight weeks after chorda tympani nerve section, none of the groups had significantly more TUJ1-positive innervation of taste buds (C). Each of the three control genotypes ($Bdnf^{lox/+}$, CreER:$Bdnf^{lox/+}$, $Bdnf^{lox/-}$, all n = 3) regained more TUJ1-positive fibers per taste bud than did mice lacking *Bdnf* (CreER:$Bdnf^{lox/-}$ n = 3), which failed to regain TUJ1-positive innervation (D). *p < 0.05, **p < 0.01, ***p < 0.001.

by nerve sectioning ($F_{(1,36)} = 739$, $p < 0.001$), which did not recover to normal levels 8 weeks after nerve section ($p = 0.001$ for all genotypes, Fig. 4B). However, all mice with normal *Bdnf* expression (*Bdnf*$^{lox/+}$, CreER:*Bdnf*$^{lox/+}$, *Bdnf*$^{lox/-}$) displayed increases in the volume of P2X3-positive fibers compared with 2 weeks post-surgery ($p = 0.001$ for all three genotypes, Fig. 4B). In mice where *Bdnf* was substantially reduced, only three taste buds in one animal showed P2X3-positive innervation, and P2X3-positive fiber volume did not significantly increase compared with 2 weeks after nerve section ($p = 0.81$, Fig. 4B). P2X3-positive fiber volume was also significantly smaller in these mutants compared with the other three genotypes ($p = 0.05$, Fig. 4B). Our results suggest that *Bdnf* deletion prevents taste buds from being fully reinnervated by a normal volume of P2X3-positive nerve fibers.

To verify findings with anti-P2X3 staining, we also counted the number of taste buds containing TUJ1-positive fibers. All taste buds from non-surgical controls contained TUJ1-positive fibers, irrespective of genotype (Fig. 4C). Nerve sectioning reduced the percentage of taste buds that contained TUJ1-positive fibers ($F_{(1,40)} = 802$, $p < 0.001$). Specifically, two weeks after chorda tympani nerve section, only 50% of the remaining taste buds had a few TUJ1-positive fibers ($p < 0.001$ for all genotypes). Eight weeks after nerve section, the percentage of taste buds containing TUJ1-positive fibers was still lower than non-surgical controls ($p = 0.001$ for all genotypes, Fig. 4C), suggesting that nerve regeneration did not recover to normal levels. Many taste buds regained TUJ1-positive fibers in mice with normal levels of BDNF (*Bdnf*$^{lox/+}$ $p = 0.002$, CreER:*Bdnf*$^{lox/+}$ $p = 0.02$, Fig. 4C), which did not occur in mice with reduced levels of *Bdnf* (CreER:*Bdnf*$^{lox/}$ $p = 0.89$). Moreover, heterozygous knockouts of *Bdnf* did not fully restore TUJ1-positive reinnervation to taste buds ($p = 0.12$). Fewer taste buds exhibited TUJ1-positive innervation in mice lacking *Bdnf* compared with controls ($p = 0.01$, Fig. 4C) 8 weeks after nerve section. Thus, *Bdnf* reduction appears to prevent reinnervation of most taste buds by TUJ1-positive nerve fibers.

We also quantified the volume of TUJ1-positive fibers in reinnervated taste buds, which was reduced after chorda tympani nerve section ($F_{(1,36)} = 802$, $p < 0.001$). Specifically, two weeks after nerve section the volume of TUJ1-positive fibers was reduced by almost 85%, irrespective of genotype or tamoxifen treatment ($p < 0.001$ for all genotypes, Fig. 4D). In all four genotypes, there were reductions in TUJ1-positive fiber volume ($p = 0.001$ for all genotypes, Fig. 4D) 8 weeks after nerve section, suggesting that taste bud reinnervation was incomplete. In controls, the volume of TUJ1-positive fibers increased 8 weeks after section compared with 2 weeks after nerve section ($p < 0.001$ for all three control genotypes), which was not the case in mice with reduced *Bdnf* expression levels ($p = 0.89$, Fig. 4D). Taste buds in these mice had reduced levels of TUJ1-positive innervation compared with controls ($p < 0.001$, Fig. 4D) 8 weeks after nerve section. Thus, restoration of TUJ1-positive innervation does not occur following *Bdnf* deletion.

In summary, 2 weeks after unilateral chorda tympani nerve section, nerve and taste bud degeneration were not affected by *Bdnf* expression. Eight weeks after nerve section, reinnervation of most taste buds had occurred, but regeneration was still incomplete, even with normal levels of BDNF. Following *Bdnf* deletion, most taste buds were not reinnervated, indicating that regeneration was either severely delayed or completely prevented by the absence of BDNF. Therefore, BDNF is required for reinnervation of taste buds after nerve injury.

3.4. Non-innervated taste buds fail to regain normal size and cell number

Because taste buds are supported by gustatory nerve innervation, we wanted to determine whether taste bud size is affected by unilateral chorda tympani nerve section. Taste buds were identified by positive staining for DAPI and cytokeratin 8 (Fig. 5A,B, green), which labels many taste bud cells. Two weeks after nerve section, taste buds were smaller in all four genotypes (Fig. 5A). Eight weeks after nerve section, however, many taste buds appeared normal in size in mice with normal levels of *Bdnf*, but not in mice in which *Bdnf* had been reduced (CreER:*Bdnf*$^{lox/-}$).

We measured taste bud volume using confocal image stacks, and determined the number of cells per bud by counting DAPI-stained nuclei within a cytokeratin 8-defined border. Chorda tympani nerve section reduced taste bud size ($F_{(1,36)} = 270$, $p < 0.001$). Specifically, two weeks after nerve section, taste bud volume and cell number decreased to half of that of non-surgical controls in all four genotypes ($p < 0.001$,for both volume and number) for all genotypes, (Fig. 5C,D). These data indicate that taste buds diminish in size to the same degree, regardless of *Bdnf* levels, and that the reduction of taste bud volume may be due to a decrease in taste cell number. Many taste buds were reinnervated in mice with normal *Bdnf* levels, and there was recovery of taste bud volume over time (*Bdnf*$^{lox/+}$ $p = 0.001$, CreER:*Bdnf*$^{lox/+}$ $p < 0.001$, *Bdnf*$^{lox/-}$ $p < 0.001$). There was also recovery in the number of cells per bud (*Bdnf*$^{lox/+}$ $p = 0.01$, CreER:*Bdnf*$^{lox/+}$ $p = 0.01$, *Bdnf*$^{lox/-}$ $p = 0.01$). Taste buds in mice with reduced *Bdnf* levels (CreER:*Bdnf*$^{lox/-}$), however, showed no recovery in either taste bud volume ($p = 0.689$) or cell number ($p = 0.796$) after nerve section. There were no differences in taste bud volume among the three control genotypes, which ruled out the effects of tamoxifen or a single *Bdnf* mutation on taste bud volume (Fig. 5C,D).

There was considerable variation in taste bud size in each animal following reinnervation. We sought to determine whether variation in taste bud size can be explained by differences in reinnervation between individual taste buds in mice with normal *Bdnf* levels. To this end, we defined taste buds that regained P2X3-positive innervation as innervated taste buds, while those lacking P2X3-positive innervation were defined as non-innervated taste buds (Fig. 6A). Reinnervated taste buds were significantly larger in mice with normal *Bdnf* expression (*Bdnf*$^{lox/+}$ $p < 0.001$, CreER:*Bdnf*$^{lox/+}$, $p = 0.001$, *Bdnf*$^{lox/-}$, $p = 0.01$, Fig. 6B) and had more taste cells ($p < 0.001$ for all three genotypes, Fig. 6C) than non-innervated taste buds. While too few taste buds in mice lacking *Bdnf* (CreER:*Bdnf*$^{lox/-}$) regained sufficient innervation for statistical analysis, these taste buds also appeared larger (Fig. 6A). We conclude that *Bdnf* deletion in adult mice prevents recovery of taste bud size because these mice had fewer reinnervated taste buds.

3.5. Bdnf gene deletion does not exacerbate geniculate ganglion neuronal loss after nerve section

Because taste nerve regeneration was delayed or prevented by BDNF reduction, we sought to determine whether the loss of innervation was caused by a loss of geniculate ganglion neurons. To determine whether geniculate ganglion neuron number is affected by either nerve section or *Bdnf* deletion, Nissl-stained neurons were counted for all groups, in 50 μm thick sections, using stereological procedures (Fig. 7A,B). Overall, there was a slight decrease in geniculate ganglion cell number following nerve section ($F_{(1,40)} = 34$, $p < 0.001$). Eight weeks after nerve section, there was significant neuronal loss across genotypes ($p < 0.001$), due to reductions in *Bdnf*$^{lox/+}$ ($p = 0.025$) and *Bdnf*$^{lox/-}$ mice ($p = 0.026$) compared with non-surgical controls. Mice lacking *Bdnf* (CreER:*Bdnf*$^{lox/-}$), however, did not lose any more neurons than the three other genotypes (*Bdnf*$^{lox/+}$ $p < 0.902$, CreER:*Bdnf*$^{lox/+}$, $p = 0.947$, *Bdnf*$^{lox/-}$, $p = 0.624$; Fig. 7C). These results suggest that *Bdnf* deletion does not cause or exacerbate neuronal loss after nerve injury. Therefore, while some geniculate neurons are lost following unilateral chorda tympani nerve section, BDNF does not impact this neuronal loss, suggesting that failure of the chorda tympani nerve to regenerate in mice lacking BDNF was not due to enhanced neuronal loss.

3.6. Taste buds on the contralateral side of the tongue fail to increase size when Bdnf is deleted

Because of a possible increase in taste bud size (Guagliardo and Hill, 2007; Li et al., 2015) on the contralateral (intact) side of the tongue

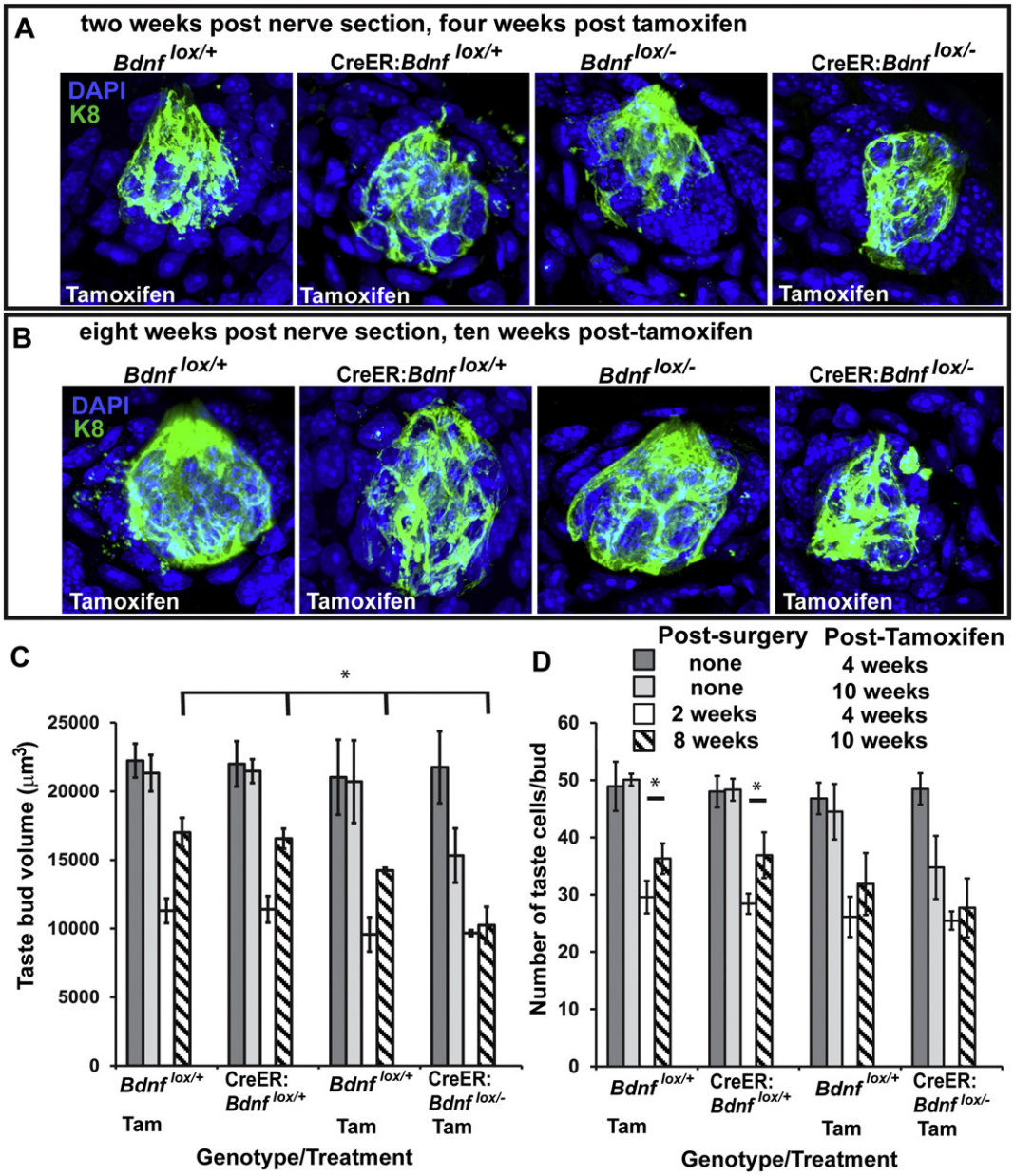

Fig. 5. Recovery of taste bud size concurrent with nerve regeneration is BDNF-dependent. Confocal images of taste buds (cytokeratin 8, green) and taste cells (DAPI, blue) in control genotypes ($Bdnf^{lox/+}$, CreER:$Bdnf^{lox/+}$, $Bdnf^{lox/-}$) and in mice with reduced *Bdnf* expression (CreER:$Bdnf^{lox/-}$) 2 (A) and 8 weeks (B) after chorda tympani nerve section. (C) Two weeks after section, the remaining taste buds were smaller than taste buds from non-surgical controls in all genotypes ($p = 0.001$, $Bdnf^{lox/+}$, CreER:$Bdnf^{lox/+}$, $Bdnf^{lox/-}$, CreER:$Bdnf^{lox/-}$, all $n = 3$). Eight weeks after section, taste bud volumes partially recovered in the three control genotypes ($Bdnf^{lox/+}$ $p = 0.001$, CreER:$Bdnf^{lox/+}$ $p = 0.001$, $Bdnf^{lox/-}$ $p = 0.001$, all $n = 3$), but not in mice with reduced *Bdnf* expression (CreER:$Bdnf^{lox/-}$ $n = 3$, $p = 0.698$). The reduction in taste bud size following nerve section is due to a loss of cells in the taste bud, because 2 weeks after nerve section, the remaining taste buds lost nearly 40% of their taste cells ($p = 0.004$), with no significant differences in this respect among the four genotypes (D). Eight weeks after nerve section, mice with normal *Bdnf* expression ($Bdnf^{lox/+}$ $n = 3$, $p = 0.016$; CreER:$Bdnf^{lox/+}$ $n = 3$, $p = 0.009$) had more taste cells per bud than at 2 weeks after section, while mice with deleted *Bdnf* ($Bdnf^{lox/-}$ $n = 3$, $p = 0.11$; CreER:$Bdnf^{lox/-}$ $n = 3$, $p = 0.431$) did not display a similar recovery. Scale bar = 10 μm and applies to all panels in A, B. *$p < 0.05$.

following nerve section, we compared each side of the tongue independently to non-surgical controls rather than comparing the sectioned side to the unsectioned side. This plasticity on the side of the tongue contralateral to nerve section could be due to the non-specific effects of surgery and/or specific effects of loss of nerve on the contralateral side. Any of these effects could involve neurotrophins, so this side of the tongue was examined independently. We first measured *Bdnf* expression in the contralateral geniculate ganglion 2, 14, 30, or 60 days after unilateral chorda tympani nerve section. *Bdnf* expression did not increase in the epithelium (Fig. 8A), but increased in the contralateral geniculate ganglion 2 weeks after unilateral nerve section ($p = 0.05$, Fig. 8B). Neurofilament expression was the same across all groups, suggesting that relatively equal numbers of neurons were isolated from each geniculate ganglion ($p = 0.05$).

Taste bud size on the unsectioned side of the tongue was compared to that of mice without nerve section for each genotype and treatment. Two weeks after the nerve section, there were no obvious differences in taste bud size or cell number on the contralateral side among the four genotypes (Fig. 8C). Eight weeks after nerve section, however, taste buds appeared larger in mice with normal *Bdnf* expression (Fig. 8C). Consistent with our previous report (Meng et al., 2015), taste buds on the unsectioned side of the tongue were smaller following *Bdnf* deletion.

To quantify these observations, taste bud volume was measured (Fig. 8D). There was an overall increase in the size of the taste bud on

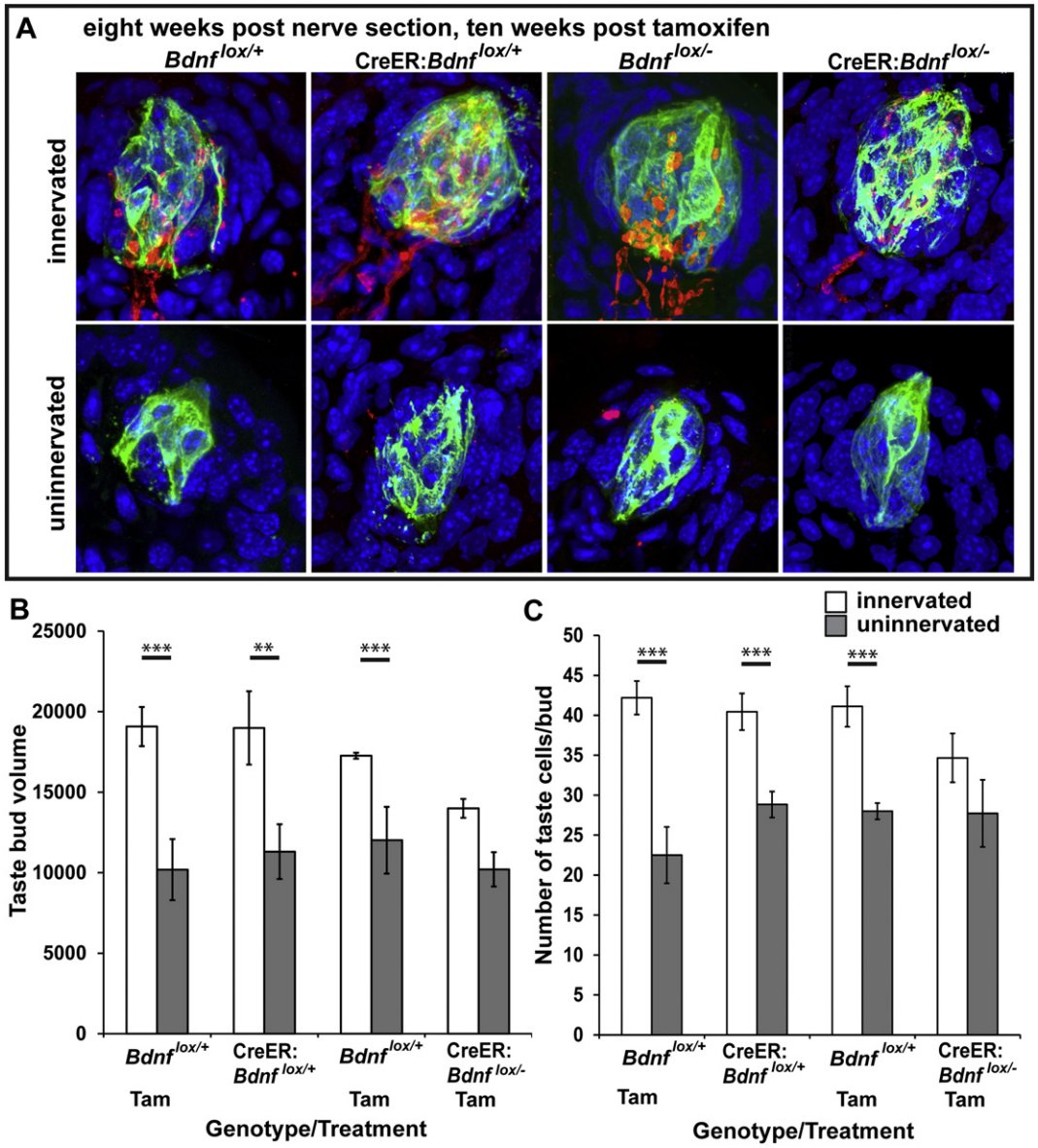

Fig. 6. Innervated taste buds are larger than non-innervated taste buds. All genotypes had both P2X3 (red) innervated (A, top) and non-innervated taste buds (A, bottom, taste = green, keratin 8, blue = DAPI for cell nuclei), albeit there were only three taste buds that regained innervation in a single animal lacking BDNF (CreER:$Bdnf^{lox/-}$). We compared the sizes of innervated versus non-innervated taste buds 8 weeks after nerve section to determine whether recovery of taste bud size is associated with reinnervation. Taste buds that were reinnervated had larger volumes than those that remained non-innervated (B). This difference in size appears to be due to differences in the number of cells per taste bud in innervated versus non-innervated taste buds (C). Scale bar = 10 μm and applies to all panels in A. $^{**}p < 0.01$, $^{***}p < 0.005$.

the contralateral side of the tongue following surgery ($F_{(1,36)} = 45$, $p < 0.001$). Two weeks after section, taste buds on the contralateral side were not different in size from non-surgical controls (Fig. 8D). Eight weeks after nerve section, however, taste bud volume increased on the contralateral side compared with non-surgical controls when *Bdnf* expression remained normal ($Bdnf^{lox/+}$ $p = 0.001$, CreER:$Bdnf^{lox/+}$ $p = 0.001$). In contrast, mice with the heterozygous *Bdnf* mutation ($Bdnf^{lox/-}$ $p = 0.057$) and mice with inducible *Bdnf* deletion (CreER:$Bdnf^{lox/-}$ $p = 0.132$) did not show statistically significant increases in taste bud volume. Therefore, the slight reduction in *Bdnf* expression in $Bdnf^{lox/-}$ mice was sufficient to prevent the increase in taste bud volume on the contralateral side. This finding indicates that normal levels of BDNF may be required for an increase in taste bud size on the contralateral side of the tongue following unilateral nerve injury. More substantial was the reduction in taste bud size 10 weeks after *Bdnf* deletion in CreER:$Bdnf^{lox/-}$ mice, both on the unsectioned side of the tongue and in non-surgical controls ($p = 0.001$, Fig. 8D). This finding replicates an earlier study demonstrating that BDNF is required to maintain normal levels of taste bud innervation (Meng et al., 2015).

To determine whether changes in taste bud size were due to changes in taste cell number, we counted the number of DAPI-stained nuclei in each taste bud. In general, we found that increases in taste bud volume on the unsectioned side were not due to an increase in cell number. Taste cell number did increase on the contralateral side compared with non-surgical controls in CreER:$Bdnf^{lox/+}$ mice ($p = 0.05$), but the effect was not as large as the increase in individual taste bud volume. Thus, the increase in taste bud volume on the unsectioned side is likely due to a combination of increased cell number and other mechanisms (i.e. cell size), rather than an increase in cell number alone. In contrast, the decrease in taste bud size in the absence of BDNF was due to a loss in taste bud cell number on the unsectioned side of the tongue ($p < 0.001$, Fig. 8E).

One possible explanation for the changes in taste bud size on the contralateral side after nerve section is altered innervation. To test this

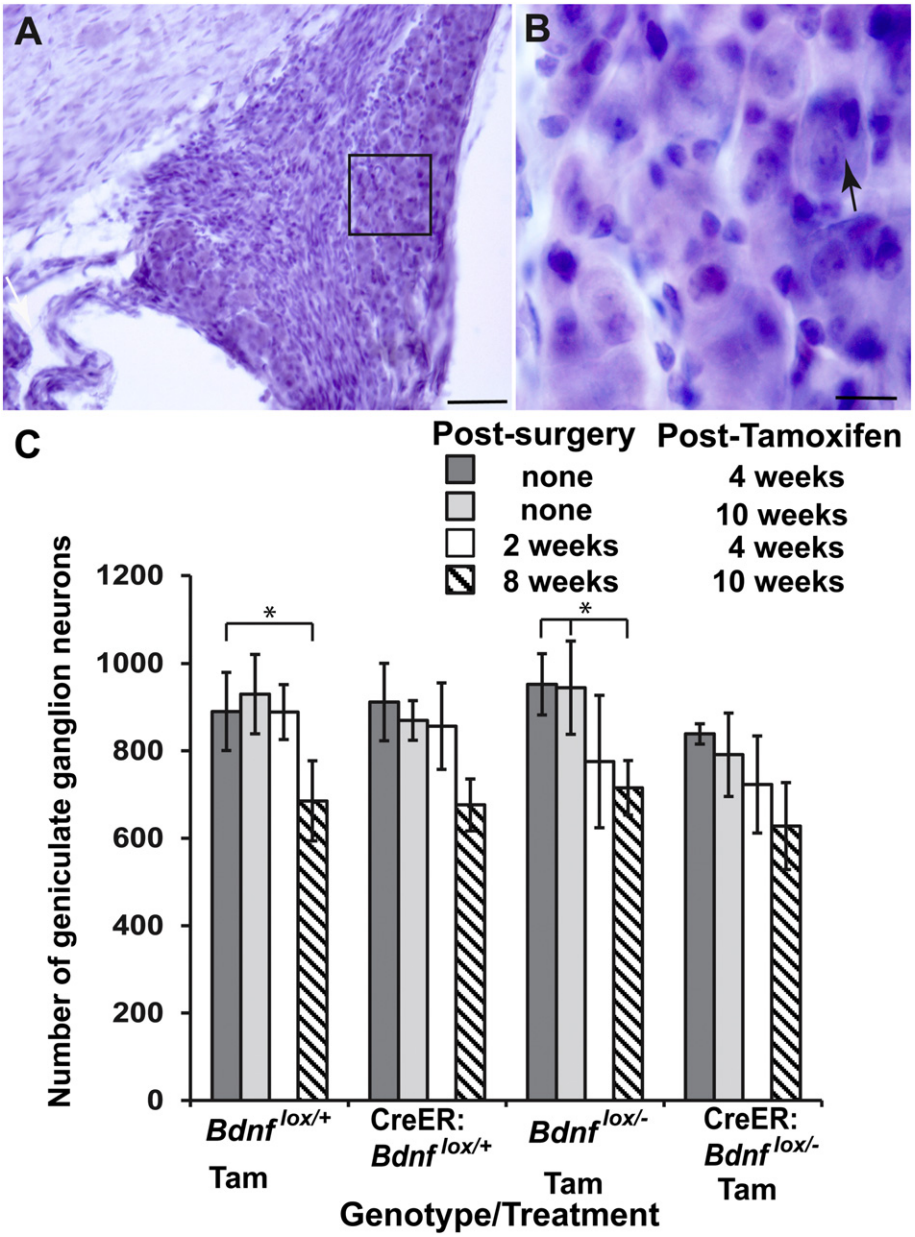

Fig. 7. BDNF does not affect geniculate ganglion neuronal loss following chorda tympani nerve section. We counted Nissl-stained geniculate ganglion neurons using stereological methods. (A) Thick (50 μm) section through the triangular-shaped geniculate ganglion stained for nissl substance, because of the section thickness neurons are both in and out of the plane of focus. (B) Pale neurons (arrow) were easily distinguished from the dark nuclei of satellite cells. Neurons were only counted when the whole neuron (top and bottom) was present in the 50-μm thick sections, such that whole neurons were quantified in a given volume of ganglion. While some geniculate ganglion neurons were lost 8 weeks after nerve section (C), this loss was not related to BDNF levels. Therefore, the failure of the chorda tympani nerve to regenerate following nerve section in mice lacking BDNF was not due to a loss of neurons. Scale bar = 50 μm in A and 10 μm in B. *$p < 0.05$.

hypothesis, we examined taste bud innervation on the contralateral side following nerve section by labeling with P2X3 (red) and TUJ1 (blue, Fig. 9A). There was no significant increase in either P2X3-positive (Fig. 9B) or TUJ1-positive (Fig. 9C) innervation in control groups 8 weeks after nerve section. This finding indicates that increased taste bud size on the contralateral side is not due to increased innervation. In mice lacking *Bdnf* (CreER:$Bdnf^{lox/-}$), there were decreases in the volume of P2X3-positive and TUJ1-positive innervation on the contralateral side of the tongue compared with the three other genotypes ($Bdnf^{lox/+}$ P2X3 $p < 0.001$, TUJ1 $p < 0.001$; CreER:$Bdnf^{lox/+}$ P2X3 $p < 0.001$, TUJ1 $p < 0.001$; $Bdnf^{lox/-}$ P2X3 $p = 0.01$, TUJ1 $p < 0.001$). Thus, the reduction in taste bud size in mice lacking BDNF is due to a loss of taste bud cells and is associated with a loss of innervation.

To summarize, unilateral chorda tympani nerve section increases taste bud size on the contralateral side of the tongue 8 weeks after nerve section. This increase is unrelated to changes in taste innervation, but may be regulated by BDNF. The role of BDNF in this process is difficult to discern largely because, in addition to supporting taste nerve regeneration, BDNF is required for maintaining normal levels of innervation to the taste bud during adulthood. These results also demonstrate that there is not a loss of innervation or taste bud size due to the non-specific effects of surgery.

4. Discussion

Gustatory neurons have robust regenerative capacities, but no factors required for their regeneration have yet been identified (Cheal and Oakley, 1977; Guth, 1957; Shuler et al., 2004; St John et al., 2003; Zalewski, 1969). Because the neurotrophin, BDNF, is required for innervation of the taste bud during normal development (Ma et al., 2009),

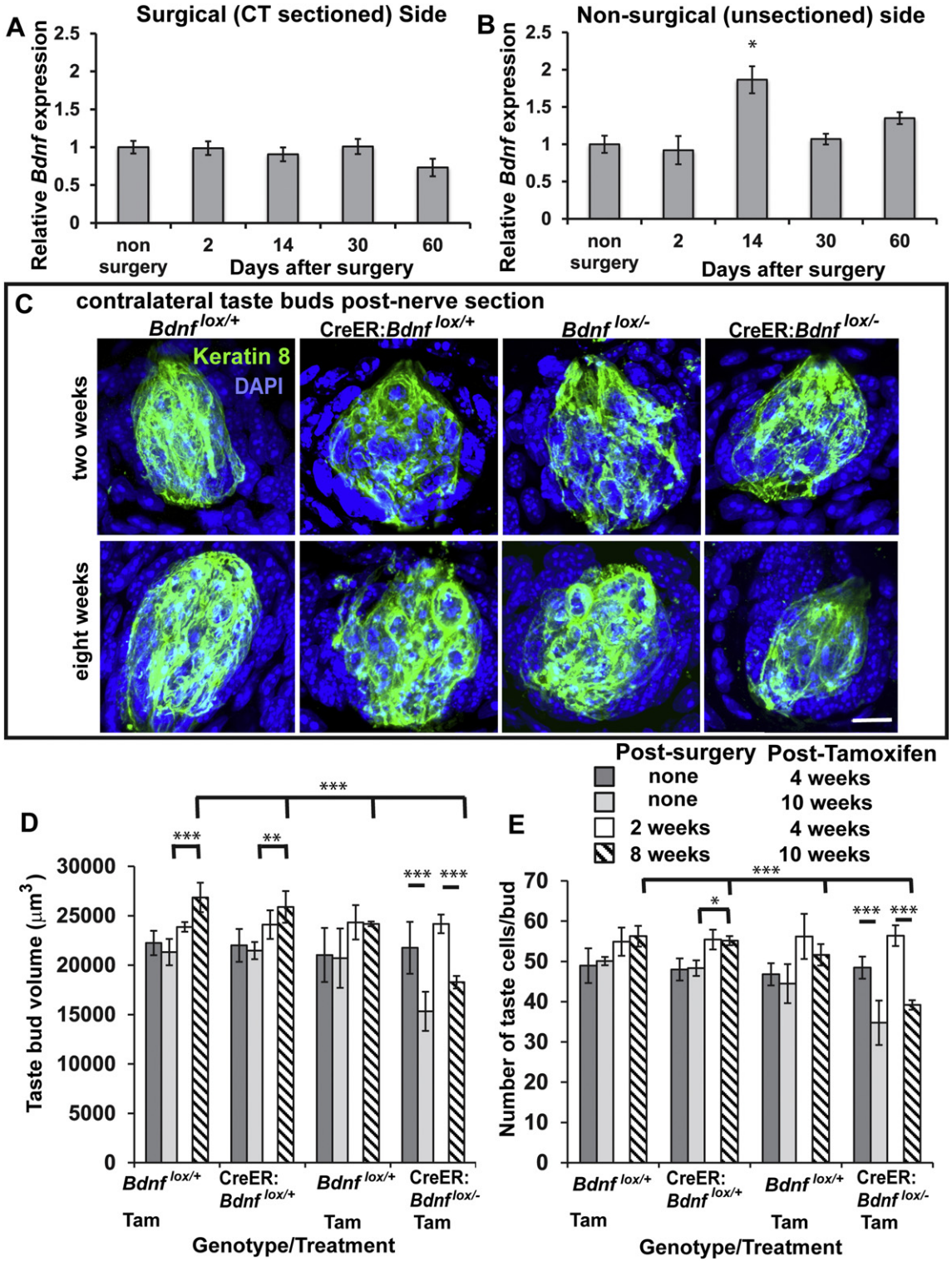

Fig. 8. Taste buds on the unsectioned side of the tongue increase in size in the presence of BDNF, but decrease in size when BDNF is reduced. Following unilateral chorda tympani nerve section, there was no change in *Bdnf* expression in the epithelium on the side contralateral to nerve section (A). The contralateral geniculate ganglion, however, displayed a significant increase in *Bdnf* mRNA expression levels14 days after section (B), suggesting that it may mediate plasticity on the contralateral side. Taste buds on the contralateral side appeared larger (C) and increased in volume (D) in $Bdnf^{lox/+}$ (n = 3) and CreER:$Bdnf^{lox/+}$ (n = 3) mice 8 weeks after nerve section compared with $Bdnf^{lox/+}$ (n = 4) and CreER:$Bdnf^{lox/+}$ (n = 4) mice that only received tamoxifen for the same length of time. Taste buds on the contralateral side decreased in size with *Bdnf* deletion (CreER:$Bdnf^{lox/-}$ n = 3) 10 weeks after tamoxifen administration regardless of whether the contra-lateral nerve had been sectioned. They also did not show an increase in either taste bud volume (D) or cell number (E), compared with non-surgical controls (n = 4). While the decrease in taste bud size following *Bdnf* removal is due to a loss of cells within the bud (E), the increase in taste bud size on the uncut side of the tongue when *Bdnf* levels are normal may not be due to a change in cell number. Scale bar = 10 μm and applies to all images in C. *p < 0.05, ** p < 0.01, ***p < 0.001.

our goal here was to determine if it is also required for reinnervation of the taste bud during regeneration. Consistent with a potential role in reinnervation, we and others (Yee et al., 2005) show that BDNF remains in the taste system following unilateral sectioning of the chorda tympani nerve. Following chorda tympani nerve section, taste buds and their innervation are lost at the same rate regardless of BDNF levels. However,

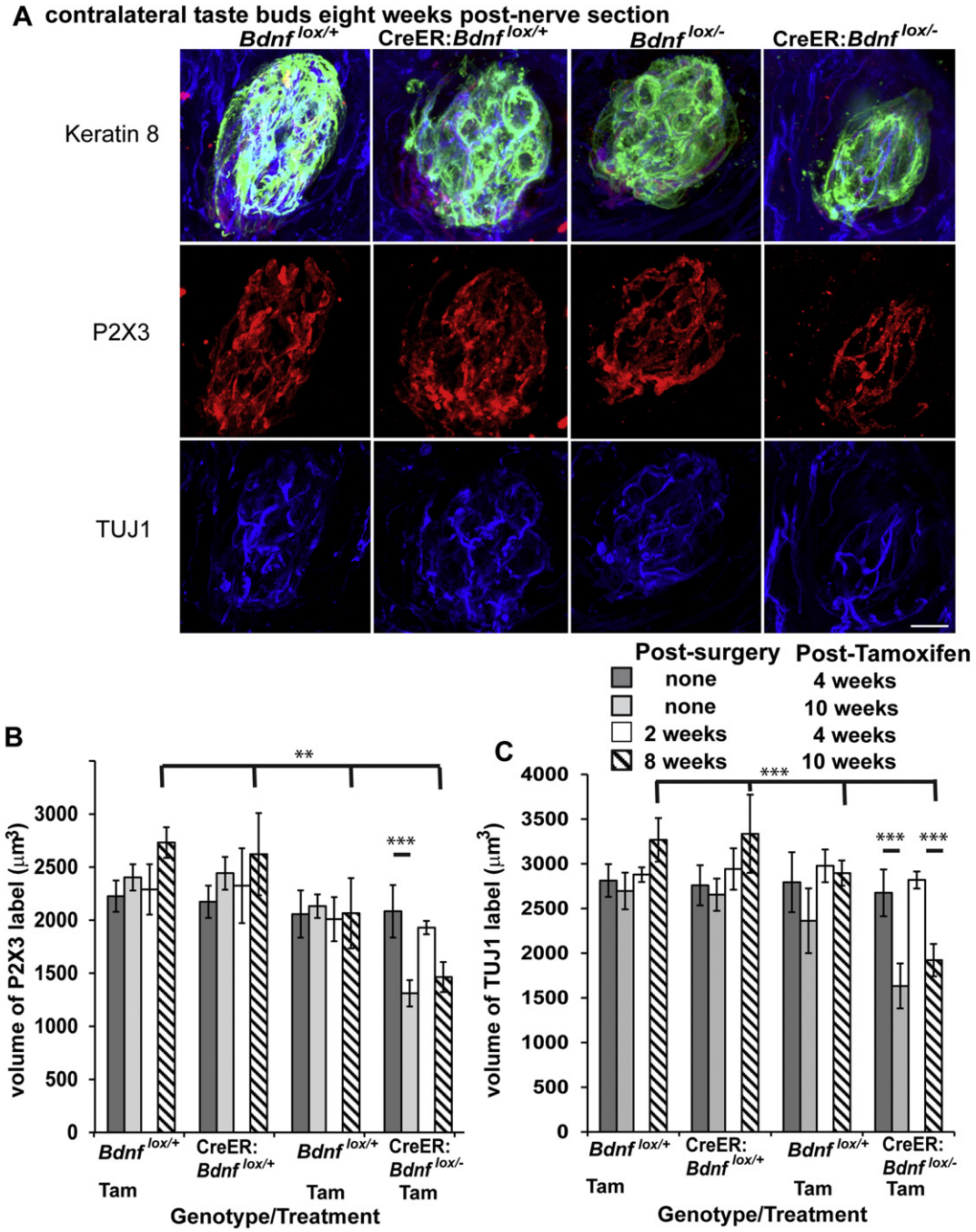

Fig. 9. *Bdnf* deletion results in a loss of innervation on the contralateral side after unilateral chorda tympani nerve section. Eight weeks after nerve section, taste buds on the contralateral side in mice lacking normal *Bdnf* appeared smaller and showed reduced innervation (A). The volume of P2X3-positive innervation decreased on the contralateral side in mice lacking *Bdnf* (CreER:$Bdnf^{lox/-}$ n = 3) compared with the three control genotypes ($Bdnf^{lox/+}$ $p < 0.001$, CreER:$Bdnf^{lox/+}$, $p < 0.001$, $Bdnf^{lox/-}$, $p = 0.01$, n = 3 all genotypes). This finding was corroborated by a reduction in the TUJ1-positive volume of innervation in the same animals ($p < 0.001$ for all genotypes). There was no consistent significant increase in innervation on the contralateral side compared with non-surgical controls in control genotypes (B, C). Therefore, the increase in taste bud size cannot be explained by an increase in innervation.

by 8 weeks post-surgery, substantial regeneration occurs in mice with normal BDNF levels resulting in the reinnervation of most taste buds. Alternatively, reinnervation of the taste bud is severely impaired in mice lacking BDNF. Therefore, BDNF is required for the chorda tympani nerve to reinnervate taste buds following deafferentation.

Although chorda tympani nerve regeneration is robust, we did not observe complete recovery 8 weeks after nerve section, even when *Bdnf* expression levels were normal. This is either because full reinnervation requires more time or does not occur. Consistent with reinnervation not recovering fully, taste bud recovery in rat reaches asymptotic levels 42 days after injury, but never reaches control levels (St John et al., 1995). Multiple factors likely impact both the speed and degree of nerve regeneration. For example, nerves regenerate more readily following a nerve crush in cat (Fugleholm et al., 1994); some gustatory studies have crushed the nerve (Cheal and Oakley, 1977; Yasumatsu et al., 2005) while others, like this study, completely transect the chorda tympani nerve (McCluskey and Hill, 2002; Shuler et al., 2004; St John et al., 1995), which could reduce the degree and rate of recovery. We found that neurons were lost in the geniculate ganglion following nerve section, consistent with previous findings in rat that fewer

geniculate neurons project via the chorda tympani to innervate the tongue following regeneration (Shuler et al., 2004). Neuron loss will limit the number of neurons available to reinnervate taste buds. Even though reinnervation did not reach control levels, half of the missing taste buds returned, and more than half of the taste buds were reinnervated by eight weeks after surgery. This fairly robust reinnervation of the taste bud is more than sufficient for functional recovery (St John et al., 1995; Yasumatsu et al., 2003; Yasumatsu et al., 2005).

While *Bdnf* deletion disrupted chorda tympani nerve regeneration, innervation was restored to a few taste buds. Individual differences in the effectiveness of *Bdnf* gene recombination, leading to some animals producing low levels of BDNF, may account for a few cases of reinnervated taste buds following *Bdnf* gene recombination. Eight weeks after nerve section, there is a large difference in the number of reinnervated taste buds between mice lacking BDNF (7.8%) and mice normally expressing *Bdnf* (70%). Therefore, even if reinnervation continued in both groups, regeneration in the mice lacking BDNF did not catch up to control levels. Given the severity of its effect on regeneration, and the fact that inducible gene recombination in adulthood is never complete, we conclude that chorda tympani nerve regeneration is dependent on BDNF.

Application of exogenous neurotrophins can enhance nerve regeneration in some cases of nerve injury (Gordon, 2009; Klimaschewski et al., 2013). Because most neurotrophic factor knockout mice die during development (Huang and Reichardt, 2001), the role of endogenous neurotrophins in nerve regeneration has been difficult to study. A few studies demonstrate that the sciatic nerve requires BDNF to regenerate (Wilhelm et al., 2012; Zhang et al., 2000). This is more likely due to the motor than the sensory component of this nerve because premotor activity stimulates BDNF production in the soma of growing motor axons, which aids regeneration (Sabatier and English, 2015). It is less clear whether sensory axons require neurotrophins for regeneration (Diamond et al., 1992), particularly because sensory neurons vary considerably in their neurotrophic requirements during development (Huang and Reichardt, 2001). Here, we found that BDNF is required for the regeneration of gustatory neurons, which are sensory.

Differences in baseline BDNF expression may underlie variation in regenerative capacity. For example, the chorda tympani nerve is routinely sectioned during middle ear surgery, and despite its robust capacity to regenerate, recovery of gustatory function does not occur in all patients (Saito et al., 2016). Neurotrophin levels also decline with age, and aging profoundly delays/prevents functional recovery after gustatory nerve injury (He et al., 2012). Disease conditions known to reduce neurotrophin levels (Gardiner et al., 2008) may also hamper peripheral nerve recovery after injury. Understanding which factors are required for peripheral nerve regeneration may help explain variation in recovery from nerve injury. The presence of at least some BDNF is critical for successful regeneration, as we report here. Therefore, exogenous BDNF may be useful during therapy following nerve injury in systems or individuals with low baseline BDNF.

Nerve regeneration is a complicated process, coordinated by numerous factors at different stages. Mice lacking BDNF had the same number of geniculate neurons available to reinnervate taste buds as mice with normal BDNF levels. Thus, the chorda tympani nerve did not fail to regenerate because of enhanced neuronal death due to *Bdnf* deletion, consistent with the concept that adult neurons do not depend on neurotrophins for survival (Angeletti et al., 1971; Easton et al., 1997; Goedert et al., 1978). Therefore, failed regeneration in mice lacking BDNF is not due to a loss of neurons. Consistently, nerve section did not increase *Bdnf* expression in the geniculate ganglion neurons, and so it is unlikely that the ganglion is an important source for BDNF during regeneration. Since BDNF expression in the neurons is not elevated, increased BDNF expression in gustatory nerves during regeneration (Yee et al., 2005), is likely due to increased levels in Schwann cells (Richner et al., 2014). So, Schwann cells could be a source of BDNF allowing gustatory neurons to regrow across the injury site (Wilhelm et al., 2012). During development BDNF from the target is required for initial innervation of the taste bud (Ma et al., 2009). We also found that BDNF continues to be produced in the lingual epithelium at normal levels, and BDNF is required to maintain innervation to the taste bud, even without injury. Therefore, taste bud-derived BDNF is likely required for reinnervation of the taste bud.

Taste buds are supported by gustatory innervation in adulthood (Guth, 1957; Cheal and Oakley, 1977; Oakley et al., 1990). Two weeks after unilateral chorda tympani nerve section, 50% of fungiform taste buds were lost, and those remaining were smaller in size. These findings are consistent with those of previous studies (Guagliardo and Hill, 2007; Whitehead et al., 1987). Following regeneration, we found that taste buds that regained innervation were normal in size, while those that did not remained small, regardless of BDNF levels. Thus, taste buds are smaller after nerve section in mice lacking BDNF because they were not reinnervated, and not because of a direct effect of BDNF on taste bud size.

In the gustatory system, nerve fibers from the contralateral side of the tongue do not typically cross the midline and innervate the denervated side of the tongue following nerve section (Kinnman and Aldskogius, 1988). However, the uncut side does show some plasticity. Specifically, there is increased taste bud size on the contralateral side following unilateral chorda tympani nerve section (Guagliardo and Hill, 2007; Li et al., 2015). For this reason, we did not use the uncut side as a control for the sectioned side, but instead examined it independently. We replicated the increase in taste bud size on the contra-lateral side of the tongue in mice expressing normal levels of BDNF; however, the effect was relatively small. It was difficult to determine if BDNF had a role in this effect because BDNF results in a decrease in innervation and taste bud size in non-surgical control conditions (Meng et al., 2015), and this effect was also evident on the contra-lateral side of the tongue. None of the genotypes showed a loss of innervation on the contra-lateral side of the tongue following surgery compared with non-surgical controls, indicating that the non-specific effects of surgical manipulation do not reduce taste bud innervation.

In conclusion, following unilateral chorda tympani nerve section, BDNF expression is increased in remaining BDNF-expressing taste bud and lingual epithelial cells. BDNF in the lingual epithelium stimulates reinnervation of the target. Once nerve fibers reinnervate the epithelium, they release a yet undefined factor, which stimulates differentiation of new taste cells, and taste buds return to their normal size.

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